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FOR THE RECORD

# HIV-1 Nef protein: Purification, crystallizations, and preliminary X-ray diffraction studies

P. FRANKEN,<sup>1</sup> S. AROLD,<sup>1</sup> A. PADILLA,<sup>1</sup> M. BODEUS,<sup>2</sup> F. HOH,<sup>1</sup> M.P. STRUB,<sup>1</sup> M. BOYER,<sup>1</sup>  
M. JULLIEN,<sup>1</sup> R. BENAROUS,<sup>2</sup> AND C. DUMAS<sup>1</sup>

<sup>1</sup>Centre de Biochimie Structurale, UMR C9955 CNRS, U414 INSERM, Université Montpellier I, Faculté de Pharmacie,  
Avenue C. Flahault, 34060 Montpellier, France

<sup>2</sup>ICGM, INSERM U332, 24 Rue du Fg St Jacques, 75014 Paris, France

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**Abstract:** Human immunodeficiency virus Nef protein accelerates virulent progression of AIDS by its interaction with specific cellular proteins involved in cellular activation and signal transduction. Here we report the purification and crystallization of the conserved core of HIV-1<sub>LAI</sub> Nef protein in the unliganded form and in complex with the wild-type SH3 domain of the p59<sup>fyn</sup> protein-tyrosine kinase. One-dimensional NMR experiments show that full-length protein and truncated fragment corresponding to the product of HIV-1 protease cleavage have a well-folded compact tertiary structure. The ligand-free HIV-1 Nef<sub>core</sub> protein forms cubic crystals belonging to space group P23 with unit cell dimensions of  $a = b = c = 86.4$  Å. The Nef–Fyn SH3 cocrystals belong to the space group P6<sub>1</sub>22 or its enantiomorph, P6<sub>5</sub>22, with unit cell dimensions of  $a = b = 108.2$  Å and  $c = 223.7$  Å. Both crystal forms diffract to a resolution limit of 3.0 Å resolution using synchrotron radiation, and are thus suitable for X-ray structure determination.

**Keywords:** crystallization; HIV-1; Nef; SH3 domain; Src kinase family

The human and simian immunodeficiency virus *nef* gene is an important determinant of virulence in experimental infection of adult monkeys and human disease (Trono, 1995). A *nef*-deleted mutant of SIV that does not cause disease in adult macaques has been used successfully as a vaccine against challenge with pathogenic virus. The critical role of Nef in human infection by HIV-1 virus was supported by studies of long-term nonprogressing subjects infected with attenuated virus strains manifesting deletions in the *nef* gene virus. The HIV-1 *nef* gene product is a 206-amino acid

N-terminal myristoylated protein of 27 kDa, found associated to the plasma membrane of infected cells. Currently, three putative functions can conceivably account for the effect of Nef: down-regulation of the surface expression of CD4, the principal viral receptor in T cells, modulation of the T cell activation status, and enhancement of viral growth rate and infectivity. The molecular mechanisms by which these actions are accomplished remain elusive, but the latter two functions rely crucially on the presence of a strictly conserved proline-rich (PxxP) motif recognized by Src homologous 3 (SH3) domains. Nef has been shown to bind to a subset of SH3 domains of the Src family of protein-tyrosine kinases (Lee et al., 1995; Saksela et al., 1995; Collette et al., 1996).

Two different models have been reported for the three-dimensional structure of HIV-1 Nef protein: the crystal structure of Nef<sub>core</sub> complexed with the Fyn SH3 R96I mutant (Lee et al., 1996) and the NMR model of unliganded Nef core domain (Grzesiek et al., 1997). We here describe the overproduction in *Escherichia coli*, the purification of HIV-1<sub>LAI</sub> Nef protein, and its further crystallization in a form suitable for medium resolution X-ray crystallographic studies of both unliganded and SH3 liganded Nef. The structure of Nef free and liganded to Fyn SH3 will allow us to accurately describe the structural changes of Nef upon ligand binding and will likely lend insight into affinity and specificity of SH3 binding.

We originally attempted to crystallize the full-length Nef HIV-1 protein, but this was unsuccessful. We therefore explored limited proteolysis in order to circumvent aggregation and define domain boundaries in the protein, which would allow us to obtain a stable molecule suitable for crystallization experiments. One-dimensional NMR data show clear evidence for significant role of ionic strength for the stability and folding of full-length and deletion mutant Nef proteins. DLS experiments were also performed to screen for monodispersity of solutions (Ferré-d'Amaré & Burley, 1994).

**Expression and purification:** Various cDNA encoding Nef LAI isolate from HIV-1 were amplified using the full-length GST-Nef cDNA (Benichou et al., 1994) as template. The resulting PCR

Reprint requests to: Christian Dumas, Centre de Biochimie Structurale, Faculté de Pharmacie, 15, Avenue C. Flahault, 34060 Montpellier, France; e-mail: dumas@cbs.univ-montp1.fr.

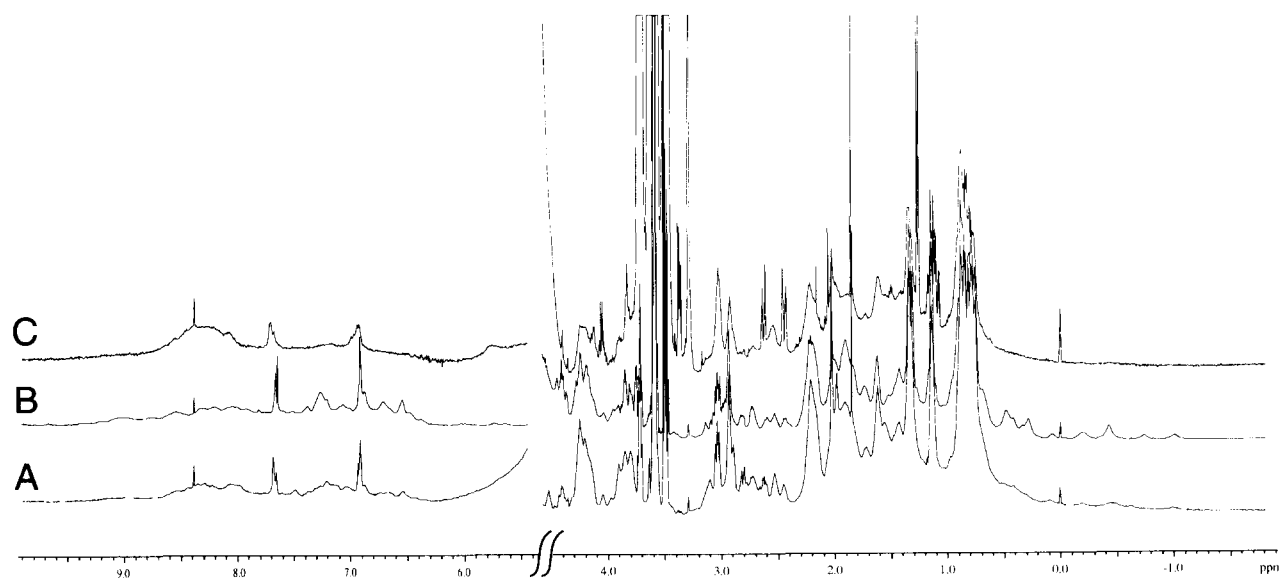
**Abbreviations:**  $\beta$ OG,  $\beta$ -octyl  $\alpha$ -D-glucoside; CMC, critical micellar concentration; DLS, dynamic light scattering; DTE, dithioerythritol; GST, glutathione S-transferase.

fragments were purified, then ligated into the *Bam*H I-*Eco*R I restriction sites of the pGEX-2T expression vector (Pharmacia). These plasmids, corresponding to Nef wild type, Nef $_{\Delta 1,57}$ , Nef $_{\Delta 1,40}$  and Nef $_{\Delta 1,20}$  mutants, were transformed into BL21(DE3) *E. coli* cells. Cultures for overproduction were grown in LB medium at 37 °C and induced with 0.5 mM isopropyl  $\beta$ -D-thiogalactopyranoside for 3 h. All recombinant Nef proteins were purified as follows: the cells were harvested, washed, and sonicated in TBS buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl), 2 mM DTE, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, and 0.4 mg/mL lysozyme (chicken egg white, Sigma). Cell debris was pelleted by centrifugation (30 min at 40,000  $\times g$ ) at 4 °C. The supernatant was applied to an ion exchange chromatography column (DEAE-FF, Pharmacia) and eluted with a gradient of 100–600 mM NaCl. The fractions containing the fusion protein were pooled and applied to a glutathione-sepharose column (Pharmacia) in TBS buffer. After extensive washing of the resin with 1 M NaCl in TBS buffer, the Nef protein was cleaved from the fused GST on the column matrix by overnight treatment at room temperature with 10 units of thrombin per milligram of fusion protein. Fractions containing the Nef protein were pooled and concentrated using Centricon concentrators (Amicon). A final gel filtration chromatography on Sephacryl S100 column (Pharmacia) yielded more than 95% pure protein. Nef protein solutions were concentrated to 10 mg/mL in 150 mM NaCl, 0.2 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 5 mM DTE, stored at 4 °C, and subsequently used for crystallization and DLS experiments. The selenomethionine-substituted Nef $_{\Delta 1,57}$  protein was overexpressed by transforming the same expression vector directly into the methionine auxotroph  $\beta$ 180 $\Delta$ metA::cat strain (Richaud et al., 1993) and purified using similar procedures as for native protein. Protein concentrations were determined by UV absorbance using calculated extinction coefficients. The identity and purity of recombinant Nef proteins were assessed by amino-terminal sequence analysis and electron-spray mass spectroscopy. Approximately 5 mg of >95% pure Nef protein were obtained per liter of culture.

The SH3 domain from Fyn tyrosine kinase, corresponding to residues 72–146 of the human enzyme (Roche et al., 1995), was expressed as GST-fusion protein in a pGEX-2T *E. coli* system. The Fyn SH3 domain was purified as described above for GST-Nef fusion proteins and concentrated to 15 mg/mL.

**Characterization of Nef by limited proteolysis, NMR, and DLS:** Recombinant HIV-1 protease (MRC AIDS Directed Program Reagent Project) and trypsin (Sigma) were used for limited proteolysis experiments. Digestion of the full-length Nef protein by trypsin and HIV-1 protease yields fragments of 19 and 17 kDa, respectively, in agreement with previously published limited proteolysis experiments on a different Nef isolate (Freund et al., 1994).

Spectroscopic experiments were performed to obtain preliminary structural information and to evaluate correct folding of the wild-type Nef and Nef $_{\Delta 1,57}$  mutant corresponding to the product of the HIV-1 protease cleavage. The 600-MHz 1D-NMR spectra recorded at 27 °C in 10 mM Hepes buffer, pH 7.2, 150 mM NaCl show that the full-length Nef protein and the Nef $_{\Delta 1,57}$  mutant have a stable and well-defined tertiary structure (Fig. 1A,B). Several broad resonances are upfield shifted to about  $-1$  ppm, indicating that methyl groups are buried in a hydrophobic environment. Amide protons are found from 7 to 9 ppm and some of them are still observed in D<sub>2</sub>O solvent and are therefore involved in hydrogen bonds or are buried in the hydrophobic core of the protein. The spectrum in Figure 1A shows broad linewidths on the order of 40 Hz, consistent with protein aggregation or oligomerization in solution. The spectrum of the mutant shows sharper linewidths than the wild-type Nef, on the order of 20–30 Hz (Fig. 1B). The spectrum recorded without salt (Fig. 1C) is characteristic for a random-coil protein because most of the amide proton resonances are found at 8.3 ppm and the methyl resonances give a unique peak centered at 0.9 ppm. DLS experiments of full-length Nef revealed aggregation; the size distribution (Boyer et al., 1996) was broad and irregular. Elimination of the amino-terminal domain



**Fig. 1.**  $^1\text{H-NMR}$  spectra of HIV-1<sub>LAI</sub> Nef protein recorded at 600 MHz in H<sub>2</sub>O at 27 °C. The protein solutions contained 0.3 mM Nef in 10 mM Hepes buffer, pH 7.2, and 150 mM NaCl. **A:** Full-length Nef protein. **B:** Nef core domain (Nef $_{\Delta 1,57}$ ). **C:** Full-length Nef protein in 10 mM Hepes buffer, pH 7.2, without NaCl.

resulted in a reduction of aggregation with a unimodal distribution centered about 8 nm.

**Crystallizations:** Crystallization conditions could only be found for Nef<sub>Δ1,57</sub> corresponding to the core domain of Nef obtained by cleavage with the HIV-1 protease. For crystal growth, a 0.4 mM Nef<sub>Δ1,57</sub> protein solution was mixed with an equal volume of reservoir solution containing 18–20% (w/v) PEG 4000 (Fluka), 5% (v/v) isopropanol, 5 mM DTE, 1 CMC βOG, over a pH range of 7.3–7.7 in 50 mM Hepes buffer. The best crystals (0.3 × 0.3 × 0.8 mm) were obtained with the sitting drop technique, where 10-μL drops were suspended over a 1-mL reservoir at 18 °C for 2–4 weeks.

Nef-Fyn SH3 cocrystals were obtained by the vapor diffusion technique at 18 °C. A Nef solution at 6.5 mg/mL in 10 mM Tris-HCl buffer, pH 8.0, with 150 mM NaCl, 0.2 mM EDTA, and 5 mM DTE is mixed with Fyn SH3 protein in the same storage buffer at a 0.7 to 0.9 SH3/Nef molar ratio, in the presence of 1 CMC βOG as additive. Two microliters of this solution are mixed with 2 μL of the well solution consisting of 30 mM bicine buffer at pH 8.9, 2.2–2.6 M NaCl, and 4% (v/v) isopropanol. Single hexagonal crystals grew to typical sizes of 0.5 × 0.5 × 0.3 mm<sup>3</sup> within 1 week.

**Preliminary X-ray studies:** Crystals of unliganded Nef<sub>core</sub> belong to cubic space group P23 with unit cell dimensions  $a = b = c = 86.4$  Å. The volume of the unit cell is consistent with a single molecule in the asymmetric unit, corresponding to a packing density ( $V_M$ ) of 3.1 Å<sup>3</sup>/Da and a solvent content of about 61%. The unit cell dimensions of hexagonal cocrystals for Nef-SH3 complex are  $a = b = 108.2$  Å and  $c = 223.7$  Å, the space group assigned to P6<sub>1</sub>22 or its enantiomorph, P6<sub>5</sub>22. The packing density ( $V_M = 3.7$  Å<sup>3</sup>/Da) corresponds to a solvent content of 67% assuming two Nef-SH3 complexes in the asymmetric unit.

Both crystal forms diffract to 3.0 Å resolution using synchrotron radiation (beamline D2AM, ESRF, Grenoble, France). However, severe radiation damage made it necessary to establish cryocooling conditions. Good results were obtained by serial transfer of these crystals in drops containing increasing amounts of glycerol (5, 10, 20, 30%) in mother liquid.

**Conclusion:** We have established crystallization and cryocooling protocols for unliganded Nef<sub>Δ1,57</sub> and in complex with the wild-type Fyn SH3 domain that enabled us to collect data suitable for X-ray structure determination. NMR experiments showed that a

minimum ionic force is required for a correct folding of Nef protein. Amino-terminal truncation of the protein improved monodispersity of solutions as shown by DLS experiments.

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## References

- Benichou S, Bomsel M, Bodeus M, Durand H, Douté M, Letourneur F, Camonis J, Benarous R. 1994. Physical interaction of the HIV-1 Nef protein with β-COP, a component of non-clathrin-coated vesicles essential for membrane traffic. *J Biol Chem* 269:30073–30076.
- Boyer M, Roy MO, Jullien M. 1996. Dynamic light scattering study of precrystallizing ribonuclease solutions. *J Crystal Growth* 167:212–220.
- Collette Y, Dutartre H, Benziane A, Ramos-Morales F, Benarous R, Harris M, Olive D. 1996. Physical and functional interaction of Nef with Lck. HIV-1 Nef-induced T-cell signaling defects. *J Biol Chem* 271:6333–6341.
- Ferré-d'Amaré AR, Burley SK. 1994. Use of dynamic light scattering to assess crystallizability of macromolecules and macromolecular assemblies. *Structure* 2:357–359.
- Freund J, Kellner R, Houthaeve T, Kalbitzer HR. 1994. Stability and proteolytic domains of Nef protein from human immunodeficiency virus type 1. *Eur J Biochem* 221:811–819.
- Grzesiek S, Bax A, Clore GM, Hu JS, Kaufman J, Palmer I, Stahl SJ, Tjandra N, Wingfield PT. 1997. Refined solution structure and backbone dynamics of HIV-1. *Protein Sci* 6:1248–1263.
- Lee CH, Leung B, Lemmon MA, Zheng J, Cowburn D, Kuriyan J, Saksela K. 1995. A single amino acid in the SH3 domain of Hck determines its high affinity and specificity in binding to HIV-1 Nef protein. *EMBO J* 14:5006–5015.
- Lee CH, Saksela K, Mirza UA, Chait BT, Kuriyan J. 1996. Crystal structure of the conserved core of HIV-1 Nef complexed with a Src family SH3 domain. *Cell* 85:931–942.
- Richaud C, Mengin-Lecreux D, Pochet S, Johnson EJ, Cohen GN, Marlière P. 1993. Direct evolution of biosynthetic pathways: Recruitment of cysteine thioethers for constructing the cell wall of *Escherichia coli*. *J Biol Chem* 268:26827–26835.
- Roche S, Fumagalli S, Courtneidge S. 1995. Requirement for Src family protein tyrosine kinases in G2 for fibroblast cell division. *Science* 269:1567–1569.
- Saksela K, Cheng G, Baltimore D. 1995. Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef+ viruses but not for down-regulation of CD4. *EMBO J* 14:484–491.
- Trono D. 1995. HIV accessory proteins: Leading roles for the supporting cast. *Cell* 82:189–192.